Established Monolayer Differentiation of Mouse Embryonic Stem Cells Generates Heterogeneous Neocortical-Like Neurons Stalled at a Stage Equivalent to Midcorticogenesis

Title: Established monolayer differentiation of mouse embryonic stem cells generates heterogeneous, neocortical-like neurons stalled at a stage equivalent to mid-corticogenesis.

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Graphical Abstract

By assessing multiple positive and negative markers of forebrain progenitors and neurons, the authors show that now-standard, partially directed neocortical differentiation of mouse embryonic stem cells generates neurons that most closely resemble neocortical projection neurons during mid-corticogenesis. These neurons are “stalled” in subtype-specific molecular refinements, as compared to primary, dissociated E15.5 neocortical neurons cultured under the same *in vitro* conditions.

(58 words; 50 max; Graphical Abstract Text filetype in ScholarOne Manuscripts)

Abstract

Two existing and widely applied protocols of embryonic stem (ES) cell differentiation have been developed to enable *in vitro* generation of neurons resembling neocortical projection neurons in monolayer culture (Gaspard *et al.*, 2008; Espuny-Camacho *et al.* 2013) and from embryoid bodies (Eiraku *et al.*, 2008; Nasu *et al.*, 2012). The monolayer approach offers advantages for detailed *in vitro* characterizations and potential mechanistic and therapeutic screening.

We investigated whether mouse ES cells undergoing largely undirected neocortical differentiation in monolayer culture recapitulate progressive developmental programs of *in vivo* progenitor and post-mitotic differentiation, and whether they develop into specific neocortical subtypes. We find that ES-derived mitotic cells that have been dorsalized by the sonic hedgehog antagonist cyclopamine, and that express, as a total population, cardinal markers of telencephalic progenitors, are, in fact, molecularly heterogeneous. We next show that these progenitors subsequently generate small numbers of heterogeneous neocortical-like neurons that are “stalled” at an immature stage of differentiation, based on multiple developmental criteria.

While some aspects of neocortical development are recapitulated by existing protocols of ES cell differentiation, these data indicate that mouse ES-derived neocortical progenitors are both more heterogeneous than their *in vivo* counterparts, and seemingly include many incorrectly specified progenitors. Further, these ES-derived progenitors spontaneously differentiate into sparse, incompletely and largely imprecisely differentiated, neocortical-like neurons that fail to adopt specific neuronal identities *in*

vitro. These results provide both foundation and motivation for refining and enhancing directed differentiation of clinically important neocortical projection neuron subtypes.

Introduction

Neocortical projection neurons undergo distinct molecular refinements at progenitor (Molyneaux *et al.*, 2005; Chen *et al.*, 2005; Chen *et al.*, 2008; Azim *et al.*, 2009) and post-mitotic (Weimann *et al.*, 1999; Arlotta *et al.*, 2005; Alcamo *et al.*, 2008; Britanova *et al.*, 2008; Lai *et al.*, 2008; Joshi *et al.*, 2008; Azim *et al.*, 2009; Tomassy *et al.*, 2010; Cederquist *et al.*, 2013) stages of development. These molecular refinements individually represent distinct developmental programs that, in sequential combinations, control neocortical development. In the absence of these critical transcriptional regulators that control any of these stages, the precise molecular identity, laminar/area positioning, and/or projection patterns of neocortical projection neuron subtypes are disrupted *in vivo*. These transcriptional controls, therefore, are good candidates for rigorous characterization of *in vitro* neocortical-like neurons derived from embryonic stem (ES) cells.

Recent advances in mouse ES cell directed neocortical differentiation recapitulate some, but not all, aspects of corticogenesis (Gaspard *et al.*, 2008; Eiraku *et al.*, 2008; Nasu *et al.*, 2012; Hansen *et al.*, 2011). Importantly, populations of ES-derived neocortical-like neurons sequentially express single genes characteristic of neocortical neurons *in vivo*. However, many of these genes (*e.g.*, Pax6, Ctip2, Satb2) are not specific only to the neocortex, but are expressed in other regions of the developing neural tube. For example, Pax6 is differentially expressed throughout the rostro-caudal extent of the neural tube ventricular zone (Ericson *et al.*, 1997; Osumi *et al.*, 1997; Briscoe *et al.*, 2000; Alaynick *et al.*, 2011), and Ctip2 is also expressed in

striatum, olfactory bulb, and hippocampus (Leid *et al.*, 2004; Arlotta *et al.*, 2005; Arlotta *et al.*, 2008).

With deeper analysis, using multiple markers, it is increasingly apparent that ES-derived neocortical-like neurons are incompletely specified *in vitro*. First, a substantial fraction of these neurons express combinations of molecular markers that are not described in the neocortex *in vivo* (*e.g.*, Reelin/Ctip2; Gaspard *et al.*, 2008). Second, ES-derived neocortical neurons often display mixed subtype-specific molecular characteristics, such as co-expression of deep- and superficial-layer markers in individual hES-derived neurons (Mariani *et al.*, 2012; Shi *et al.*, 2012). Finally, these neurons display skewed areal specification and projection patterns to visual and limbic targets (Gaspard *et al.*, 2008; Espuny-Camacho *et al.*, 2013). These subtle but distinct deficiencies in the differentiation of ES-derived neocortical neurons suggest incomplete differentiation, which might hinder neocortical subtype acquisition, and limit the interpretability of these *in vitro* models of corticogenesis.

More refined characterizations of *in vitro* neocortical differentiation are now possible, given recent advances in the study of neocortical development (Molyneaux *et al.*, 2007; Woodworth *et al.*, 2012; Custo Greig *et al.*, 2013). Pax6, often used to exclusively mark the pallium, is not a specific marker of the pallial tissue, given its expression throughout the neural tube (Alaynick *et al.*, 2011). In the absence of positional information *in vitro*, characterization of Pax6-expressing “pallial” progenitors is incomplete without the presence of additional markers of pallial progenitors (*e.g.*, Sox6; Azim *et al.*, 2009; Otx2, Acampora *et al.*, 1999), or the absence of other markers co-expressed with Pax6 outside of the pallium.

Sox6 is a transcription factor that controls the development of pallial progenitors independently from Pax6; its absence results in misspecification of pallial progenitors, by ectopic expression of subpallial genes (Azim *et al.*, 2009). Like Pax6, Sox6 is not specific to the pallium, as it is also expressed by post-mitotic, subpallium-derived interneurons. However, when Sox6 is assessed in combination with Pax6, the presence of both markers greatly increases the specificity for pallial progenitors. To date, this combination has not been used for the identification of pallial progenitors *in vitro*.

Post-mitotic neocortical neurons *in vivo* undergo a prolonged maturation process, during which gene expression becomes progressively restricted to particular subtypes (Lai *et al.*, 2008; Britanova *et al.*, 2008; Alcamo *et al.*, 2008; Chen *et al.*, 2008; Azim *et al.*, 2009; Joshi *et al.*, 2008; Woodworth *et al.*, 2012; Cederquist *et al.*, 2013; Custo Greig *et al.*, 2013). These neurons initially co-express transcriptional controls characteristic of multiple neocortical projection subtypes (*e.g.*, Tbr1, Ctip2, Satb2, Clim1, Lmo4) and multiple neocortical area identities (*e.g.*, CoupTF1, Bhlhb5, Lmo4) before distinct subtype identities emerge. Together, this process of molecular refinement involves, at minimum, coordinated neuronal maturation, neocortical projection neuron class distinction, and neocortical area subtype distinction. These three stage-specific features of neocortical identity refinement form the basis for our approach to characterizing neocortical identity *in vitro*, presented here.

We assessed mouse ES cell-derived neocortical-like neurons at progenitor and post-mitotic stages, and identified multiple characteristics consistent with stalled maturation. First, ES-derived neocortical-like progenitors are more heterogeneous than has been previously reported using single-marker analyses. Second, neocortical-like

neurons are stalled at a maturation stage resembling mid-corticogenesis, as indicated by overlapping expression of multiple subtype-specific markers that do not resolve with time. Additionally, area-specific differentiation is abnormal, as ES-derived neocortical neurons are deficient in the sensori-motor cortex regulator of neocortical development, Bhlhb5. Overall, this approach rigorously investigates the refinement of ES-derived neocortical differentiation, and indicates directions for refining directed differentiation of clinically important neocortical projection neurons.

Materials and Methods

Cell culture and differentiation

Murine embryonic stem cells: Nagy ES cell line G4 (MMRRC stock# 011987-MU) or feeder-free E14Tg2a (Baygenomics) mouse embryonic stem cells were propagated using standard procedures (Ying *et al.*, 2003) on gelatin-coated (0.1% gelatin, StemCell Technologies) cell culture treated plastic dishes. Nagy ES cells were cultured on mouse embryonic fibroblast feeder cells (Millipore EmbryoMax PMEF-N). Mouse embryonic stem cell media is GMEM (Invitrogen) supplemented with 10% ESC-certified fetal bovine serum (vol/vol, Invitrogen), 0.1 mM nonessential amino acids (Invitrogen), 1mM sodium pyruvate (Invitrogen), 0.1mM β-mercaptoethanol (Sigma), 50 U/mL penicillin/streptomycin and 1000 U/mL leukemia inhibitor factor (ESGRO).

For differentiation, Nagy G4 and E14Tg2a ES cells were plated at low density (5,000 cells/cm^2) on gelatin-coated plastic dishes in ES cell medium, and cultured as described (Gaspard *et al.*, 2009). Briefly, ESCs were trypsinized, dissociated, and plated on gelatin-coated cell culture plates. Medium was changed to DDM after one day. DDM consists of DMEM/F12 (Invitrogen-Gibco) supplemented with N2 supplement (N2 supplement consists of 8.61uM insulin, 1mM transferrin, 2uM progesterone, 10mM putrescine and 3uM selenite; Invitrogen-Gibco), 2mM glutamine, 0.1 mM nonessential amino acids, 1mM sodium pyruvate, 0.5 mg/ml bovine serum albumin fraction V (all from Invitrogen-Gibco), and 0.1mM β-mercaptoethanol (Sigma).

Cyclopamine (Calbiochem) or Ag1.3 (gift from Lee Rubin, Harvard University) was added from day 2 to day 10 in the differentiation medium at a final concentration of 1uM. After 10 to 14 days of differentiation, cells were trypsinized, dissociated and plated on poly-lysine/laminin (Becton-Dickinson) coated glass coverslips, and allowed to grow for 4–14 days in N2B27 medium. N2B27 medium consists of a 1:1 mixture of DDM and Neurobasal that is supplemented with B27 (without vitamin A; Invitrogen-Gibco) and 2 mM glutamine.

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde (wt/vol) for 30 min, and washed three times in phosphate-buffered saline (PBS). Wide-field image acquisition was performed using a Nikon 90i epifluorescence microscope with a Clara DR-328G cooled CCD digital camera (Andor Technology, Belfast, Northern Ireland). Confocal imaging was performed with a BioRad Radiance 2100 Rainbow laser-scanning confocal microscope based on a Nikon E800 microscope. Images were assembled in Adobe Photoshop and Illustrator (CS3, CS5), with adjustments for contrast, brightness, and color balance to obtain optimal visual reproduction of data.

Table of Primary Antibodies used

Antigen	**Immunogen**	**Manufacturer, species, mono- vs. polyclonal, catalog number**	**Dilution used**
Pax6	QVPGSEPDMSQYWPRLQ derived from the C-terminus of mouse Pax-6 protein	Covance, rabbit polyclonal, #PRB-278P	1:300
Sox6	synthetic peptide derived from <800 residues to the C-terminus of mouse SOX, conjugated to KLH	Abcam, rabbit polyclonal, #AB30455	1:200

Mash1	full length recombinant rat Mash1 protein	BD, mouse monoclonal, #556604	1:500
Gsh2	synthetic peptide derived from a region between amino acids 1-46 of human GSH2	Abcam, rabbit polyclonal, #26255	1:500
Nkx2.1/TTF1	synthetic peptide containing residues 110-122 at the N-terminus of rat Nkx2.1	BioPat, mouse monoclonal, #PA0100	1:5,000
Tbr1	synthetic peptide derived from within residues 50-150 of mouse TBR1, conjugated to KLH	Abcam, rabbit polyclonal, #31940-100	1:500
Ctip2	synthetic protein derived from within residues 1-150 of human CTIP2	Abcam, rat monoclonal [25B6], #18465-100	1:500
Satb2	recombinant human Satb2 protein containing a fragment of the C-terminal	Abcam, mouse monoclonal [SATBA4B10], #51502	1:200
Er81	synthetic mouse Er81 C-terminal peptide sequence, CNPHPYNEGYVY, conjugatd to KLH	Abcam, rabbit polyclonal, #AB36788	1:100
GAD67	recombinant GAD67 protein	Millipore, mouse monoclonal [clone 1G10.2], #MAB5406	1:1000
TuJ1/β-tubulin III	synthetic peptide containing residues 441-450 of human β-tubulin III (Ala446 to Ser446 substitution) with N-terminal added cysteine, conjugated to KLH	Sigma, rabbit polyclonal, #T2200-200uL	1:1000
TuJ1/β-tubulin III	microtubules derived from rat brain	Covance, mouse monoclonal, #MMS-435P	1:1000
Map2	full length recombinant bovine Map2	Sigma, mouse monoclonal, #M1406	1:500
NeuN/Fox-3	purified cell nuclei from mouse brain; recognizes residues 1-106 of Fox-3	Millipore, mouse monoclonal [A60], #MAB377	1:250
Bhlhb5	synthetic protein containing the N-terminus of hamster Bhlhb5	Santa Cruz, goat polyclonal, #6045	1:300
CoupTF1	synthetic protein containing the first 203 residues of mouse COUP-TFI	rabbit polyclonal, gift of the Michele Studer lab, Institute of Biology Valrose in Nice, France	1:500

Antibody Characterization

Please see **Table 1** for a list of all antibodies used.

The Pax6 antiserum has been widely characterized and used in the field, and does not label Pax6 null cells. It is confirmed to be specific to the developmental pallial domain by immunocytochemistry.

The Sox6 antiserum does not stain samples of post-natal brain from a Sox6 knockout mouse (manufacturer's data sheet). Identical results in embryonic brain tissue were obtained from the Macklis laboratory (Azim et al., Nat. Neurosci., 2009).

The Mash1 antiserum recognizes a single band of 34 kDa molecular weight by SDS-PAGE (per manufacturer), with specific staining of the mouse subpallium (Yun, Fischman, et al., Development 2002).

The Gsh2 antiserum recognizes a single band of 35 kDa molecular weight by SDS-PAGE (per manufacturer) and is confirmed to be specific to the developmental subpallial domain by immunocytochemistry.

The Nkx2.1/TTF1 antiserum staining is abolished when the diluted primary antibody is pre-incubated with 0.1uM of the immunizing peptide (Moreno and Gonzalez, 2007) and is confirmed to be specific to the developmental subpallial domain by immunocytochemistry.

The Tbr1 antiserum recognizes a single band of 74 kDa molecular weight by SDS-PAGE and stained a pattern of cellular morphology and distribution in

the mouse brain that is identical with previous reports (Hevner et al., Neuron, 2001)

The Ctip2 antiserum detects two bands representing Ctip2 at about 120kD by SDS-PAGE. No staining is seen on tissue from a Ctip2 knockout mouse (Arlotta et al., Neuron, 2005; Arlotta et al., J. Neurosci. 2008).

The Satb2 antiserum detects one 81kDa band representing Satb2 by SDS-PAGE. No staining is seen on tissue from a Satb2 knockout mouse (Britanova et al., Neuron 2008).

The Er81 antiserum produced a pattern of immunoreactivity that was identical with previous descriptions of E16.5 mouse brain sections (Stenman *et al.*, 2003; Yoneshima *et al.*, 2006).

The GAD67 antiserum had no detectable cross reactivity with GAD65 by Western blot on rat brain lysate (manufacturer's data sheet).

The TuJ1/ β-tubulin III antisera is well characterized and highly reactive to neuron specific class III β-tubulin (βIII). TuJ1 does not identify β-tubulin found in glial cells.

The MAP2 antiserum localizes the high molecular weight forms of MAP2 (MAP2a and MAP2b) but shows no reactivity with MAP2c (manufacturer's data sheet). No cross-reactivity is observed with MAP1, MAP5, tubulin, or tau.

The NeuN (Neuronal Nuclei) antiserum recognizes two major bands of 40 and 50 kDa by SDS-PAGE. The antiserum recognizes residues 1-106 at the N-terminal of Fox-3. It is specific to two Fox-3 isoforms, based on absent

NeuN staining in Fox-3 null SK-N-SH cells and identical co-localization of Fox-3 and NeuN antisera (Kim, Adelstein, and Kawamoto, JBC 2009).

The Bhlhb5 antiserum does not stain tissue from a Bhlhb5 knockout mouse (data not shown, Joshi et al., Neuron 2008).

The CoupTF1 antiserum did not stain tissue from a CoupTF1 knockout mouse (Tripodi et al., Development 2004; Srubek Tomassy et al., PNAS 2010).

Secondary antibodies were from the Invitrogen Molecular Probes Alexa series. Specificity was tested with omission of primary antibodies (data not shown).

Nuclei were stained with Hoechst #33342 (1:3,000, Sigma).

Mice

All mouse studies were approved by the Harvard University and/or Massachusetts General Hospital IACUCs, and were performed in accordance with institutional and federal guidelines. The date of vaginal plug detection was designated E0.5, and the day of birth as P0. Wild-type CD1 mice were used in all experiments (Charles River Laboratories).

Brains were fixed using standard methods (Fricker-Gates *et al.*, 2002; Arlotta *et al.*, 2005). Briefly, brains were fixed by trans-cardial perfusion with PBS–heparin (10 U/ml), followed by 4% paraformaldehyde, and post-fixed overnight at 4°C in 4% paraformaldehyde. Brains were sectioned coronally at 50 um on a vibrating microtome (Leica). Coverslips or floating sections were blocked in 1% BSA (Sigma) and 0.1%

Triton X-100 (Sigma) for 20min at room temperature, before incubation in primary antibody.

Results

To begin characterizing ES-derived neocortical-like cells, we cultured mouse ES cells, and directed their differentiation to neocortical fates using an established monolayer cell culture protocol (Gaspard *et al.*, 2008; Gaspard *et al.*, 2009). This protocol enables rostral and dorsal differentiation by plating ES cells at low-density, removing serum and retinoids, and antagonizing residual Shh morphogen signaling with cyclopamine. We replicated this protocol, and generated sequential waves of broad neural populations (neural progenitors, immature neurons, and astroglia) from E14Tg2a mouse ES cells over the course of 28 days (**Figure 1**), closely replicating the originally published results (Gaspard *et al.*, 2008). After two weeks in culture, 55 +/- 6.8% (mean +/- s.e.m.) of ES-derived cells express Nestin, an intermediate filament protein, broadly marking neural progenitors. Similar results were obtained using Nagy G4 mouse ES cells (data not shown). These results show that both the timing of neural induction, and sequential generation of neural progenitors, neurons, and astroglia, are nearly identical to previously published results (Gaspard *et al.*, 2008).

Distinct subsets of pallial progenitors are generated from ES cells

We first assessed the proportion of rostral, dorsal, pallial-like differentiation by ES-derived Nestin-expressing neural progenitors at *in vitro* day 14. Approximately half of Nestin-expressing progenitors are pallial-like, based on co-expression of Pax6 (**Figure 2A**). All Pax6-expressing cells co-express Nestin, and Pax6 is not expressed by any TuJ1 (beta tubulin III)-expressing immature neurons (data not shown). Together,

these data suggest that Pax6 expression is restricted to about half of ES-derived neural progenitors.

We next asked whether these Pax6-expressing neural progenitors at *in vitro* day 14 display other characteristics of pallial progenitors. We hypothesized that correctly-specified pallial progenitors will co-express Sox6 (Azim *et al.*, 2009). Similar to the proportion of Pax6-expressing progenitors, we find that approximately half of Nestin-expressing progenitors also express Sox6 (**Figure 2B**). However, Pax6 and Sox6 are co-expressed by only approximately 20% of progenitors (**Figure 2C**), which is strikingly dissimilar to their highly overlapping expression *in vivo* (Azim *et al.*, 2009). Overall, the combined distribution of Pax6 and Sox6 expression accounts for the majority of Nestin-expressing progenitors at day 14, but these pallial transcription factor controls are largely not expressed by the same cells.

Since most Pax6-expressing cells do not co-express Sox6, we hypothesized that some Pax6-expressing cells might possess identities characteristic of a position in the neural tube caudal to the telencephalon. Otx2, expressed throughout the ventricular zone of the neural tube rostral to the hindbrain, demarcates the midbrain-hindbrain boundary, and is required for early specification of forebrain and midbrain (Acampora *et al.*, 1999). We find that the majority of Pax6-expressing pallial-like progenitors co-express Otx2, consistent with a forebrain progenitor identity (**Figure 2D**). Otx2 is also co-expressed by most Sox6-expressing progenitors (**Figure 2E**). While these data suggest that many Pax6- and Sox6-expressing progenitors resemble forebrain pallial progenitors, the absence of Otx2 co-expression in many progenitors indicates further heterogeneity not observed *in vivo*.

To assess whether downstream pallial molecular programs are intact in cells differentiating under these conditions, we assessed expression of Ngn2 in these ES-derived pallial-like progenitors. In the developing pallium, Pax6 and Sox6 are both upstream of Ngn2, a pro-neurogenic transcription factor that has cell cycle dependent expression in progenitors undergoing neurogenesis (Azim *et al.*, 2009; Kageyama *et al.*, 2008; Ma *et al.*, 2008; Schuurmans *et al.*, 2004). We find that Ngn2 is highly expressed by cells with low Pax6 expression, suggesting that ES-derived pallial-like progenitors are undergoing neurogenesis with dynamic regulation of Pax6 and Ngn2 (**Figure 2F**).

To investigate whether pallial-like progenitors appropriately exclude markers of subpallial identity, we tested for molecular markers of these populations at *in vitro* day 14. Mash1, also called Ascl1, is a transcription factor expressed in the subpallium (both lateral and medial ganglionic eminences) and at the adjacent pallial-subpallial boundary; in concert with Dlx1/2, it is essential for the proper specification of subpallium-derived neurons (Long *et al.*, 2009). We find that Mash1 is co-expressed by approximately 10% of Nestin-expressing ES-derived progenitors (**Figure 2G**).

To investigate whether Mash1-expressing progenitors display other characteristics of subpallial progenitors, we assessed their co-expression with Gsh2 and/or Nkx2.1. Gsh2 is a transcription factor expressed by early progenitors of the lateral ganglionic eminence, and, to a lesser extent, the medial ganglionic eminence; Gsh2 functions upstream of Mash1 activation, and represses Pax6 transcription (Corbin *et al.*, 2003; Wang *et al.*, 2009; Pei *et al.*, 2011; Azim *et al.*, 2009; Batista-Brito *et al.*, 2009). Nkx2.1 is another subpallial control, expressed in the medial ganglionic eminence (Butt *et al.*, 2008). Gsh2 and Nkx2.1 are individually co-expressed with

Mash1 in the subpallium, in distinct compartments; we hypothesized that some Mash1-expressing progenitors might co-express one or both these subpallial transcription factors. However, we find that Gsh2 and Nkx2.1 expression is absent in ES-derived progenitors (data not shown).

To determine whether this protocol is competent to generate cells with appropriate subpallial characteristics, we directed the ventralization of ES-derived neural progenitors with Shh agonism. In the presence of the Shh agonist Ag1.3, Pax6 expression is appropriately lost (**Figure 2H**), while expression of Nkx2.1 and Gsh2, individually, is increased (**Figure 2I,J**). Mash1 expression was not affected (data not shown). These data provide a positive control for the absence of Gsh2 and Nkx2.1 expression with cyclopamine-mediated dorsal differentiation, confirming that subpallial gene expression by ES-derived progenitors is Shh-dependent, as expected *in vivo*. In contrast, Mash1 expression by a subpopulation of these cells appears to be independent of subpallial specification.

We next asked whether Mash1-expressing progenitors are instead pallial-like, given previous reports of cells with Mash1 expression in the dorsal pallium and at the pallial-subpallial boundary *in vivo* (Britz *et al.*, 2006; Ge *et al.*, 2006). While pallial progenitors expressing Pax6 (**Figure 2K**) or Sox6 (**Figure 2L**) are mostly distinct from Mash1-expressing progenitors, we find that approximately 15% of Pax6-expressing progenitors co-express Mash1 (**Figure 2K**). These findings suggest that many Mash1-expressing ES-derived progenitors are potentially pallial. This interpretation is consistent with the broad dorsalization induced by cyclopamine in ES cell differentiation,

but again highlights a high degree of heterogeneity within ES-derived pallial-like progenitors by day 14.

A small subset of ES-derived neurons is neocortical, based on multiple markers

At 21 days of differentiation, 31 +/- 3.3% (mean +/- s.e.m.) of cells express TuJ1, and can be considered immature neurons, although this proportion is highly variable (**Figure 1G and 3A,B**). Previous reports using this protocol have indicated that a higher proportion of ES-derived neurons are generated (Gaspard *et al.*, 2008; Gaspard *et al.*, 2009), which raises specific methodological points that might explain the quantitative differences we observe. First, the ES-derived cells produced by this monolayer protocol do not remain a monolayer after greater than seven days of differentiation; at later times, we observe cell overgrowth and "clumping" of cells with heterogeneous morphologies. We use confocal imaging to more precisely localize TuJ1 staining near areas of dense cell over-growth at day 21. Counting total nuclei within aggregates of cells has not proved reliable, and we therefore excluded neurons found within these dense aggregates. Second, we maintained strict criteria for counting TuJ1-expressing neurons: TuJ1 staining must minimally encompass a hemi-circle around the nucleus, and display a polarized, neuron-like morphology. Third, because TuJ1 expression is not entirely specific to neurons (*e.g.*, TuJ1 is expressed by fibroblasts; Vierbuchen *et al.*, 2010), we excluded non-neuronal TuJ1-expressing cells based on multiple exclusion criteria: comparatively lower intensity of TuJ1 expression, fibroblast-like morphology, or any nuclei surrounded by an exceedingly high density of neurites from adjacent neurons, which can sometimes incorrectly resemble distinct neurons. Finally, TuJ1

expression is not distributed uniformly *in vitro* across a coverslip, and all characterizations were performed on selected imaging fields containing substantial numbers of neurons.

To investigate the potentially neocortical identity of these ES-derived neurons at day 21, we performed immunostaining for multiple neuronal markers. We first assessed the expression of Tbr1, which is expressed briefly by all post-mitotic pyramidal neurons generated in the developing pallium, before its expression becomes restricted to corticothalamic projection neurons (CThPN) and callosal projection neurons (CPN) in layer VI (Englund *et al.*, 2005; Hevner *et al.*, 2001). Tbr1 is expressed in few brain areas other than neocortex, and Tbr1-expressing neurons are glutamatergic (Hevner *et al.*, 2001; Bedogni *et al.*, 2010; McKenna *et al.*, 2011). Approximately 10-20% of TuJ1-expressing neurons *in vitro* also express Tbr1 (**Figure 3A**). Given the low percentage of ES-derived neurons expressing Tbr1, we imaged selected fields containing relatively high concentrations of Tbr1-expressing neurons for further subtype characterization.

To identify cells with properties of early neocortical neurons, and potentially of specific deep-layer subtypes, we focused on expression of Ctip2. Like Tbr1, Ctip2 is a critical transcription factor expressed at distinct levels (off, low, high) by multiple newly post-mitotic neocortical subtypes; later in development, Ctip2 controls corticofugal projection neuron (CFuPN) axon outgrowth and fasciculation, with refined laminar expression specific to deep layers – low level by CThPN in layer VI, and high level by SCPN in layer V (Arlotta *et al.*, 2005). Expression of Ctip2 by immature CPN, and therefore co-expression with CPN marker Satb2, is lost by late embryogenesis (Alcamo *et al.*, 2008; Britanova *et al.*, 2008; Chen *et al.*, 2008). Importantly, Ctip2 is highly

expressed in brain regions other than the neocortex, most highly by medium-sized spiny neurons in the striatum (Leid *et al.*, 2004; Arlotta *et al.*, 2005; Arlotta *et al.*, 2008).

We find that Ctip2, similarly to Tbr1, is expressed by a modest fraction of TuJ1-expressing neurons (approximately 10-20% of neurons in selected fields containing positive Ctip2 staining) (**Figure 3B**). If these Ctip2-expressing neurons are neocortical-like, we hypothesized that most should also express Tbr1. Consistent with this prediction, Ctip2 and Tbr1 display nearly complete co-expression after 21 days in culture (**Figure 3C**). These data suggest that this sparse population of ES-derived Ctip2-expressing neurons is glutamatergic, and most closely resembles immature deep-layer projection neurons.

To rigorously investigate whether these Ctip2 and Tbr1 co-expressing neurons represent non-neocortical neurons, we performed co-expression analysis of Ctip2 with Er81 and GAD67. Er81 is expressed in neocortical deep layers, olfactory bulb (interneurons), amygdala, thalamus, but not in striatum (Stenman *et al.*, 2003; Yoneshima *et al.*, 2006); the intersection of Er81 and Ctip2 expression is fairly exclusive to neocortex. We find that Ctip2-expressing neurons co-express Er81 in the cytoplasm (**Figure 3D**), which indicates that they are not striatal. Many important striatal genes, such as Darpp32, Foxp1, and Foxp2, are expressed both in cortex and in striatum, and we therefore examined expression of GAD67, which is expressed only by GABAergic inhibitory populations, such as medium-sized spiny neurons and subpallium-derived cortical interneurons (reviewed in Gord and Bernardo, 2011). We find that the ES-derived neurons expressing Ctip2 do not co-express GAD67, and, therefore, are not GABAergic (**Figure 3D**). Together, the co-expression of Ctip2, Er81, and Tbr1, and the

absence of GAD67, strongly support the interpretation that a small proportion of ES-derived neurons under these relatively undirected conditions adopt properties of immature neocortical neurons *in vitro*.

Neocortical neurons are relatively immature

To investigate whether neocortical-like Ctip2-expressing neurons display appropriate features of stage-specific differentiation, we first assessed basic markers of neuronal maturation. Nearly all CNS neurons activate common programs of neuronal maturation, as marked by TuJ1, Map2, and NeuN/Fox-3 (Kim *et al.*, 2009). Very few mature neurons in the CNS lack NeuN expression, most notably Purkinje neurons and gamma spinal motor neurons (Friese *et al.*, 2009).

We find that these ES-derived neurons are relatively immature, based on the low abundance of NeuN expression after 21 or 28 days (approximately 5-10% of TuJ1-positive neurons co-express NeuN, assessed in selected fields *in vitro*). Given the importance of neuronal maturation for the timing of post-mitotic neocortical subtype refinement, we asked whether the small population of neocortical-like neurons that co-express Ctip2, Tbr1, and Er81 is mature or immature. We find that all Ctip2-expressing neurons co-express TuJ1 (**Figure 3B**). Approximately one third of these neurons express both Map2 and NeuN (**Figure 4A**). These neurons are not uniformly or completely mature, but some display crucial hallmarks of at least early maturation.

We next investigated whether the extent of NeuN expression might indicate an equivalent stage in development. *In vivo* at E16.5-E18.5, NeuN is expressed by

approximately one-third of of Ctip2-expressing neocortical neurons (**Figure 4B,C**). Later, at P6, all Ctip2-expressing neocortical neurons also express NeuN (**Figure 4D**). Between E16.5 and P6, Ctip2-expressing cortical neurons *in vivo* extend axons to their targets in the midbrain, brainstem, and spinal cord, and begin the process of pruning collateral connections (Arlotta *et al.*, 2005; Stanfield, 1992). In contrast, ES-derived neocortical neurons in culture develop to a relatively immature state most highly resembling mid-corticogenesis.

Impaired subtype distinction of immature ES-derived CFuPN

We next assessed whether ES-derived, Ctip2-expressing, immature neurons are appropriately molecularly distinct from other subtypes. During mid-corticogenesis, *in vivo*, when only a small percentage of neurons express NeuN, neocortical projection neurons co-express markers characteristic of multiple subtypes. By the first week of postnatal neocortical development, this molecular co-expression resolves into a refined, subtype-specific molecular identity, termed “subtype refinement” (Lai *et al.*, 2008; Joshi *et al.*, 2008; Azim *et al.*, 2009; Cederquist *et al.*, 2013; Lickiss *et al.*, 2012). One example transcription factor, Satb2, is transiently expressed by early-stage CFuPN, but is later restricted to specific expression by CPN and other associative neocortical neurons (Alcamo *et al.*, 2008; Britanova *et al.*, 2008; Lickiss *et al.*, 2012). To specifically investigate this subtype refinement of Ctip2 and Satb2 *in vivo*, for comparison to the events in culture, we assessed E16.5 neocortex and find significant Ctip2/Satb2 co-expression in layer V; these immature Ctip2/Satb2 co-expressing post-mitotic neurons consistently do not express NeuN (**Figure 5A**).

As a further, direct comparison, we next assessed post-mitotic subtype refinement by primary developing neocortical neurons *in vitro* using dissociated E12.5 neocortical cells cultured under the same conditions as day 14-21 ES-derived neocortical neurons. We find that these primary neurons reduce their initially high levels of Ctip2 and Satb2 co-expression, and increase the intensity of either Ctip2 or Satb2 over the course of four days *in vitro* (**Figure 5B-D**), confirming that primary neurons are capable of subtype-specific transcription factor refinement during maturation *in vitro*.

We then investigated whether the small population of ES-derived, Ctip2-expressing, immature neocortical neurons similarly display molecular profiles consistent with mid-corticogenesis, and whether this molecular identity refines to subtype specificity over time. We find that most Ctip2-expressing neurons continue to co-express Satb2 at 21 days (one week after the onset of *in vitro* neurogenesis) (**Figure 5E**). Strikingly, Ctip2/Satb2 co-expression is still maintained after 28 days of post-mitotic differentiation (**Figure 5F**), in contrast to primary dissociated E15.5 neocortical neurons cultured for only four days under the same conditions *in vitro* (**Figure 4B-D**). Moreover, these ES-derived neocortical-like neurons express a continuum of low, medium, and high expression levels of Ctip2 and Satb2, in contrast to primary dissociated E15.5 neocortical neurons, which have distinctly high, low, or absent expression levels of Ctip2 or Satb2, when cultured under the same conditions *in vitro*.

Incomplete molecular area refinement of ES-derived CFuPN

It has been previously reported that some ES cell-derived neurons, when grafted in the white matter tracts ventral to the neocortex of P0/P1 mice, project axons to intra-

cortical and subcerebral (mostly visual) targets after one month (Gaspard *et al.*, 2008). The expression of a single caudal neocortex marker, CoupTF1, was used to explain these biased projection patterns. Since the time of that publication, multiple transcription factors (*e.g.*, Bhlhb5, CoupTF1, and Lmo4) have been characterized as important post-mitotic controls over neocortical area specification *in vivo* (Joshi *et al.*, 2008; Armentano *et al.*, 2007; Tomassy *et al.*, 2010; Huang *et al.*, 2009; Cederquist *et al.*, 2013). In striking parallel to initially broad expression of genes that refine over time to define precise subtype identity, these post-mitotic area controls are initially co-expressed broadly in all neocortical areas, then become refined in expression during the first postnatal week (Woodworth *et al.*, 2012; Custo Greig *et al.*, 2013).

To investigate whether immature ES-derived CFuPN might have area-specific molecular identity, we assessed putative CFuPN marked by high Ctip2 expression. We find that nearly all ES cell-derived Ctip2-expressing neurons co-express CoupTF1 (**Figure 6A**), which is consistent with *in vivo* broad expression (caudal-high to rostral-low gradient) in the neocortex at mid-corticogenesis. In striking contrast, Ctip2-expressing neurons do not co-express Bhlhb5 (**Figure 6B**), although Bhlhb5 is expressed by other ES-derived neurons (**Figure 6C**). These data indicate that the absence of Bhlhb5 co-expression is inappropriate for the same stage of development *in vivo,* and might represent deficits in area-specific differentiation by ES-derived neocortical neurons.

Discussion

The experiments presented here are the first to deeply investigate the differentiation of neocortical-like neurons derived from ES cells, using the current and rapidly advancing knowledge in the field, and the results identify maturation deficits of these neurons. We demonstrate the utility of coordinating markers of neuronal maturation with markers of neocortical subtypes to assess the stage and extent of neocortical differentiation. Previous reports of ES-derived neocortical neuronal subtypes have assessed the presence of individual markers or, less commonly, combinations of very limited and relatively broad markers to identify neocortical subtypes (Gaspard *et al.*, 2008; Eiraku *et al.*, 2008; Nasu *et al.*, 2012; Ideguchi *et al.*, 2010; Mariani *et al.*, 2012; Shi *et al.*, 2012; Espuny-Camacho *et al.*, 2013). However, most neocortical subtype-specific markers are only truly specific during transient developmental stages, in defined anatomical locations, and are not individually specific to the neocortex (Molyneaux *et al.*, 2007; Woodworth *et al.*, 2012; Custo Greig *et al.*, 2013).

Developmental stage-specific characterizations of ES-derived neocortical-like neurons *in vitro* suggest that these neurons most resemble *in vivo* immature, un-refined neocortical neurons at mid-corticogenesis. This conclusion is based on three distinct developmental criteria. First, less than one third of TuJ1-expressing neocortical-like neurons express mature neuronal markers (MAP2, NeuN), consistent with the proportion of neocortical neurons that express NeuN *in vivo* at E16.5-E18.5 (**Figure 4**). These data provide a metric for comparison to a similar developmental stage *in vivo*; we use this information to interpret the stage-specific expression of subtype markers. Second, neocortical-like neurons co-express multiple subtype-specific transcription

factors (*e.g.*, Tbr1, Ctip2, Satb2) in a continuum of low, medium, and high expression levels consistent with *in vivo* co-expression of these genes during early- to mid-corticogenesis, but which is in striking contrast to the more mature expression of these transcription factors by primary, dissociated E15.5 neocortical neurons cultured under the same conditions *in vitro* (**Figure 3; Figure 5**). Third, neocortical-like neurons appropriately co-express some, but not all, post-mitotic controls over area-specific differentiation (*e.g.*, CoupTF1, Bhlhb5; **Figure 6**); while this expression profile is most consistent with caudal fates, it does not reflect the broad patterns of area-specific markers during mid-corticogenesis.

Neocortical projection neurons are not the only population that displays increasingly restricted expression of subtype-specific transcription factors during maturation; indeed, spinal motor neurons (SMN) follow a similar process of refinement and diversity generation *in vivo* (Jessell, 2000; Dasen and Jessell, 2009; Alaynick *et al.*, 2011). Initially, early post-mitotic SMN express the transcription factors Hb9, Islet1, and Lhx3 (Sharma et. al., 1998), and with continued maturation and position-dependent differentiation (Sürmeli *et al.*, 2011), expression of each transcription factor becomes progressively restricted to distinct SMN subtype identities, including medial, lateral, and hypaxial motor column subtypes. However, *in vitro* subtype-specific molecular refinements by heterogeneous ES-derived SMN are not distinct at early, immature stages of differentiation (Wichterle *et al.*, 2002; Soundararajan *et al.*, 2006; Peljto and Wichterle, 2011). Our findings, though directed toward characterizing neocortical neuronal identities, also reveal unresolved, immature subtype refinement *in vitro*.

Though ES-derived neocortical-like neurons recapitulate some aspects of

immature neocortical development specific to a stage approximating mid-corticogenesis, these data also indicate that these neurons are “stalled” in maturation *in vitro*. This conclusion is based on the comparison of subtype refinement by primary dissociated neocortical cells and ES-derived neocortical neurons under the same culture conditions (**Figure 5**). The immature subtype marker profiles in ES-derived neurons do not resolve over the course of two weeks *in vitro*, in contrast to the timing observed *in vivo*, or to primary neurons cultured with the same conditions *in vitro*. The conclusion that ES-derived neocortical-like neurons are stalled in differentiation, rather than permanently mis-specified, is supported by evidence of continued neuronal maturation, based on the extension of long-range axons to forebrain and midbrain targets but not by resolution of subtype-specific molecular markers, following transplantation into early post-natal mice (Gaspard *et al.*, 2008).

Increasingly, more refined analyses of ES-derived neuron physiology and subtype identity indicates stalled or incomplete neuronal differentiation following directed differentiation *in vitro*. For example, in one protocol of SMN generation from mouse ES cells, *in vitro* maturation is limited; only after five days of myotube co-culture do ES-derived SMN express more mature physiologic properties of post-natal spinal motor neurons (Miles *et al.*, 2004). Recently, more detailed analyses of ES-derived photoreceptor neurons (Eiraku and Sasai), midbrain-like dopaminergic neurons (Kriks *et al.*, 2011), and spinal nociceptor neurons (Chambers *et al.*, 2012), similarly suggest variability and limitations in the extent of neuronal subtype maturation *in vitro*, and following grafting *in vivo*.

We speculate that the maturation deficits in ES-derived neocortical neurons are the result of both intrinsic and extrinsic deficits. First, recent mouse studies demonstrate that the absence of specific intrinsic factors might accelerate, delay, or interrupt mature laminar or area positioning (*e.g.*, Sox5 in Lai *et al.*, 2008; FoxG1 in Miyoshi and Fishell, 2012; Bhlhb5 in Joshi *et al.*, 2008; CoupTF1 in Tomassy *et al.*, 2010, and Alfano *et al.*, 2011). The finding that Bhlhb5 is absent in ES-derived neocortical-like neurons at mid-corticogenesis is consistent with at least one intrinsic deficit in area-specific transcriptional refinement. Second, simplified growth and media conditions *in vitro* might exclude extrinsic factors necessary for neocortical subtype distinction (reviewed in Tiberi *et al.*, 2012). Co-culturing with astrocytes might be beneficial, particularly for synaptic maturation and other refinements that occur later in postnatal development (Johnson *et al.*, 2007; Foo *et al.*, 2011), although the deficits of subtype-specific molecular refinement by ES-derived neocortical neurons occur prior to the stage that coincides with post-natal gliogenesis. Third, the absence of cell-cell interactions in adherent cell culture might impede subtype-specific refinements; strikingly, subtype marker overlap does not appear to be as severe in aggregate-based protocols of ES-derived neocortical differentiation, possibly indicating the utility of cell-cell interactions within self-organized ES-derived aggregates (Eiraku *et al.*, 2008; Nasu *et al.*, 2012). Similarly, subtype-specific maturation of ES-derived neocortical-like neurons might occur when transplanted as individually isolated neurons *in vivo,* into embryonic or post-natal neocortex, although such subtype characterizations have not been performed *in situ* (Gaspard *et al.*, 2008). Finally, intrinsic deficits in the chromatin landscape might contribute to the stalled maturation of ES-derived neocortical-like neurons; recent

studies suggest that chromatin remodeling is important at multiple stages of corticogenesis (MacDonald and Roskams, 2009; Tiberi *et al.*, 2012; Baranek *et al.*, 2012). We speculate that some of these deficits might contribute to the insufficiency of ES-derived progenitors, by multiple protocols, to generate distinct superficial-layer neuron subtypes (Hansen *et al.*, 2011).

Early deficits in pallial progenitor specification might explain the sparse enrichment and stalled maturation of post-mitotic neocortical neurons. Our data presented here describe heterogeneity of pallial and forebrain markers (*e.g.*, Pax6, Sox6, Otx2, and Mash1) and absence of subpallial markers in ES-derived progenitors (**Figure 2**). While these data suggest that dorsalization of ES-derived progenitors is highly efficient, the heterogeneity and minimally overlapping expression of multiple pallial markers (*e.g.* Pax6 and Sox6) strongly indicate an incomplete extent of pallial differentiation by most ES-derived progenitors. In particular, the strikingly low efficiency of neocortical-like neuron generation (at most 20% of ES-derived neurons express Tbr1, Ctip2, or Satb2) supports the interpretation that most ES-derived pallial-like progenitors are incompletely specified. We speculate that the small population of Pax6 and Sox6 co-expressing progenitors (~20% of total progenitors; **Figure 2C**) most closely resembles true pallial progenitors and likely accounts for the small population of neocortical-like neurons; the prospective isolation of these ES-derived pallial-like progenitors might enable further study of neocortical subtype specification in future studies. Together, these data suggest that deficits in neocortical-like neuron subtype specification might originate with incomplete pallial progenitor specification.

Judging from the typically exceptional specificity of neocortical neuronal subtype involvement with specific neurodegenerative diseases (*e.g.*, CSMN and spinal motor neurons in ALS; cortico-striatal projection neurons in Huntington's disease), the utility of directed differentiation for studying neocortical biology, pathologic mechanisms, and potential therapies likely hinges on its close approximation to *in vivo* development. While the results presented in this report suggest caution in utilizing ES-derived neocortical cells as a model for cortical development, with further refinements these protocols might be substantially improved. For example, the same protocol for ES-derived neocortical directed differentiation was recently used as a model system to identify Bcl6 as a regulator of neocortical progenitors, and this pathway was verified *in vivo* (Tiberi *et al.*, 2012). Absent a mechanistic understanding of the deficits of ES-derived neocortical neuron differentiation, these data indicate specific directions for the continued refinement of directed differentiation to more closely approximate neocortical development. For example, deficits in the transcriptional state or chromatin landscape of ES-derived neurons might be targeted for manipulation to enhance neocortical differentiation (Juliandi *et al.*, 2012).

Taken together, the data from these experiments and from prior work by other groups indicate that ES-derived neocortical differentiation is limited *in vitro*, with multiple maturation deficits not consistent with *in vivo* development. The stage-specific, multiple-marker methodology presented here promises to be increasingly useful for the characterization of neocortical subtypes, and for potentially directing the differentiation of refined subtypes. These results provide both foundation and motivation for refining,

enhancing, and enriching for directed differentiation of clinically important CFuPN as a class, and of distinct cortical projection neuron subtypes.

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Figure Legends

Figure 1. Sequential generation of neural progenitors, neurons, and astroglia in an established monolayer ES cell protocol is reproducible. (A, B) Nestin expression decreases, as a proportion of total cells, from day *in vitro* (DIV) 14 to DIV 21. (C, D) TuJ1 expression increases, as a proportion of total cells, from day 14 to day 21. (E, F) GFAP expression begins by day 28. (G) Quantification of Nestin, TuJ1, GFAP expression over the course of 28 days. Data are represented as mean +/- s.e.m. (N = 3). Scale bar: panel A, and also for panels B–F, 20um.

Figure 2. Pallial-like progenitors generated by ES-derived progenitors are molecularly heterogeneous. (A, B) Half of Nestin-expressing progenitors co-express Pax6 (A) or Sox6 (B). (C) ~20% of progenitors express both Pax6 and Sox6. (D, E) The majority of Pax6 (D) and Sox6 (E) pallial progenitors co-express Otx2. (F) Ngn2 is expressed by some Pax6 low/negative progenitors. (G) Mash1/Nestin subpallial-like progenitors represent ~10% of cells. (H-J) Ventralized ES cells lose Pax6 expression (H), and increase subpallial Nkx2.1 (I) and Gsh2 (J). (K, L) Pax6/Mash1 (K) and Sox6/Mash1 (L) cellular subsets are mostly distinct. Scale bar: panel A, and also for panels B-L, 10um. A magenta-green version of this figure is available as a supplementary figure.

Figure 3. ES-derived, Ctip2-expressing neurons are neocortical-like. (A) Tbr1-expressing neurons co-express TuJ1. (B) Ctip2-expressing neurons co-express TuJ1.

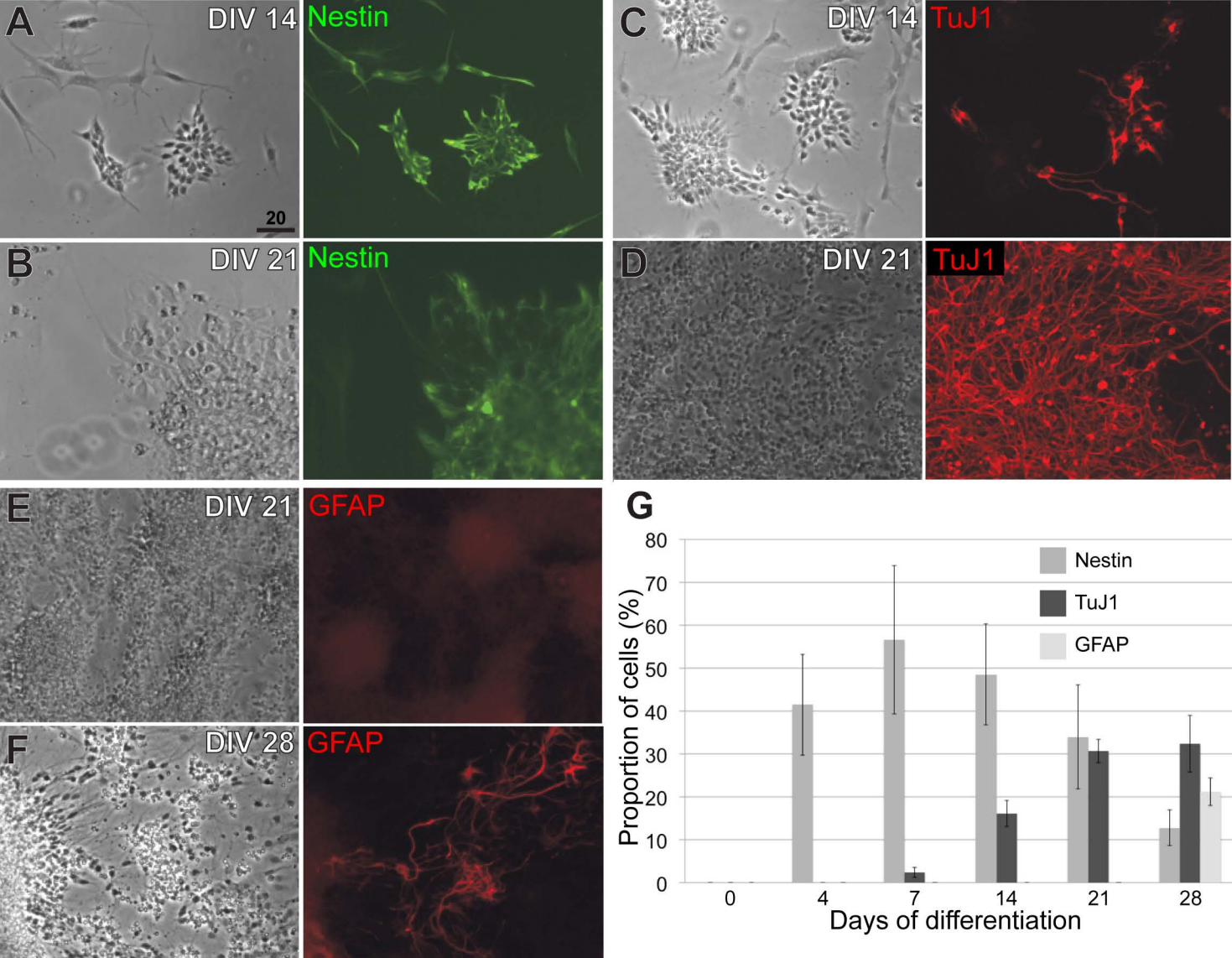
(C) Ctip2-expressing neurons co-express Tbr1. (D) Ctip2-expressing cells are distinct from GAD67-expressing cells; Er81 is co-expressed by Ctip2 neurons (filled arrowheads: Ctip2/Er81; empty arrowheads: GAD67). Scale bar: panel A, and also for panels B-D, 10um. A partial magenta-green version of this figure is available as a supplementary figure.

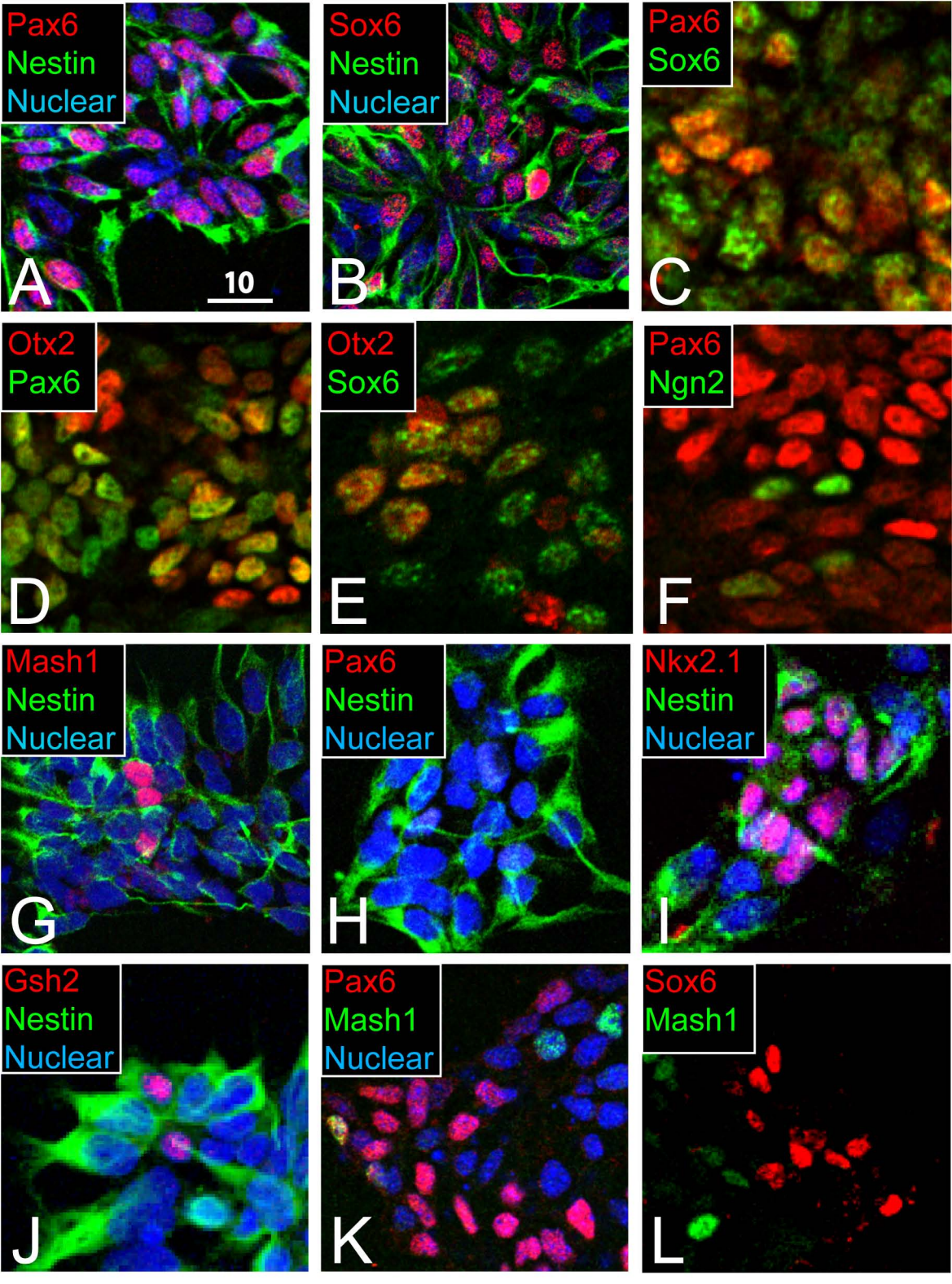
Figure 4. ES-derived, Ctip2-expressing neurons are immature, consistent with NeuN expression at mid-corticogenesis *in vivo*. (A) Approximately one third of ES-derived, Ctip2-expressing neurons co-express Map2 and NeuN *in vitro* (arrows: Ctip2/Map2/NeuN) (B,C) *In vivo,* NeuN is normally expressed by one third of (B) E16.5 and (C) E18.5 Ctip2-high neurons. (D) By P6, *in vivo*, all retrograde-labeled SCPN co-express Ctip2 and NeuN, indicating completion of a next stage of progressive maturation. Scale bars: panel A, 10um; panel B, and also for panel C, 150um; panel D, 30um. A partial magenta-green version of this figure is available as a supplementary figure.

Figure 5. ES-derived, Ctip2-expressing neurons do not resolve immature projection neuron marker expression over one week *in vitro*. (A) At E16.5 *in vivo*, Ctip2 and Satb2 co-expressing neurons are relatively immature neurons, indicated by the absence of NeuN co-labeling. (B, C, D) Dissociated primary E15.5 neocortical cells initially co-express Ctip2 and Satb2, but this immature expression resolves during the course of four days *in vitro* (DIV). (E, F) Under the same culture conditions, ES-derived neocortical-like neurons co-express Ctip2 and Satb2 at 21 days (E) and this co-

expression persists at 28 days (F). Scale bars: panel A, 150um; panel B, and also for panels C-F, 10um. A magenta-green version of this figure is available as a supplementary figure.

Figure 6. ES-derived, Ctip2-expressing neurons do not complete post-mitotic area refinements. (A) All ES-derived Ctip2-expressing neurons co-express CoupTF1. (B) All ES-derived Ctip2-expressing neurons cells exclude Bhlhb5. (C) Bhlhb5 is expressed by other ES-derived neurons (N=4; approximately 1,000 neurons were screened). Scale bar: panel A, and also for panels B-C, 10um. A magenta-green version of this figure is available as a supplementary figure.


A
DIV 14
Nestin
20
C
DIV 14
TuJ1
B
DIV 21
Nestin
D
DIV 21
TuJ1
E
DIV 21
GFAP
F
DIV 28
GFAP
G
Nestin
TuJ1
GFAP
Proportion of cells (%)
0
10
20
30
40
50
60
70
80
0
4
7
14
21
28
Days of differentiation


Pax6
Nestin
Nuclear
A
10
Sox6
Nestin
Nuclear
B
Pax6
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C
Otx2
Pax6
D
Otx2
Sox6
E
Pax6
Ngn2
F
Mash1
Nestin
Nuclear
G
Pax6
Nestin
Nuclear
H
Nkx2.1
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K
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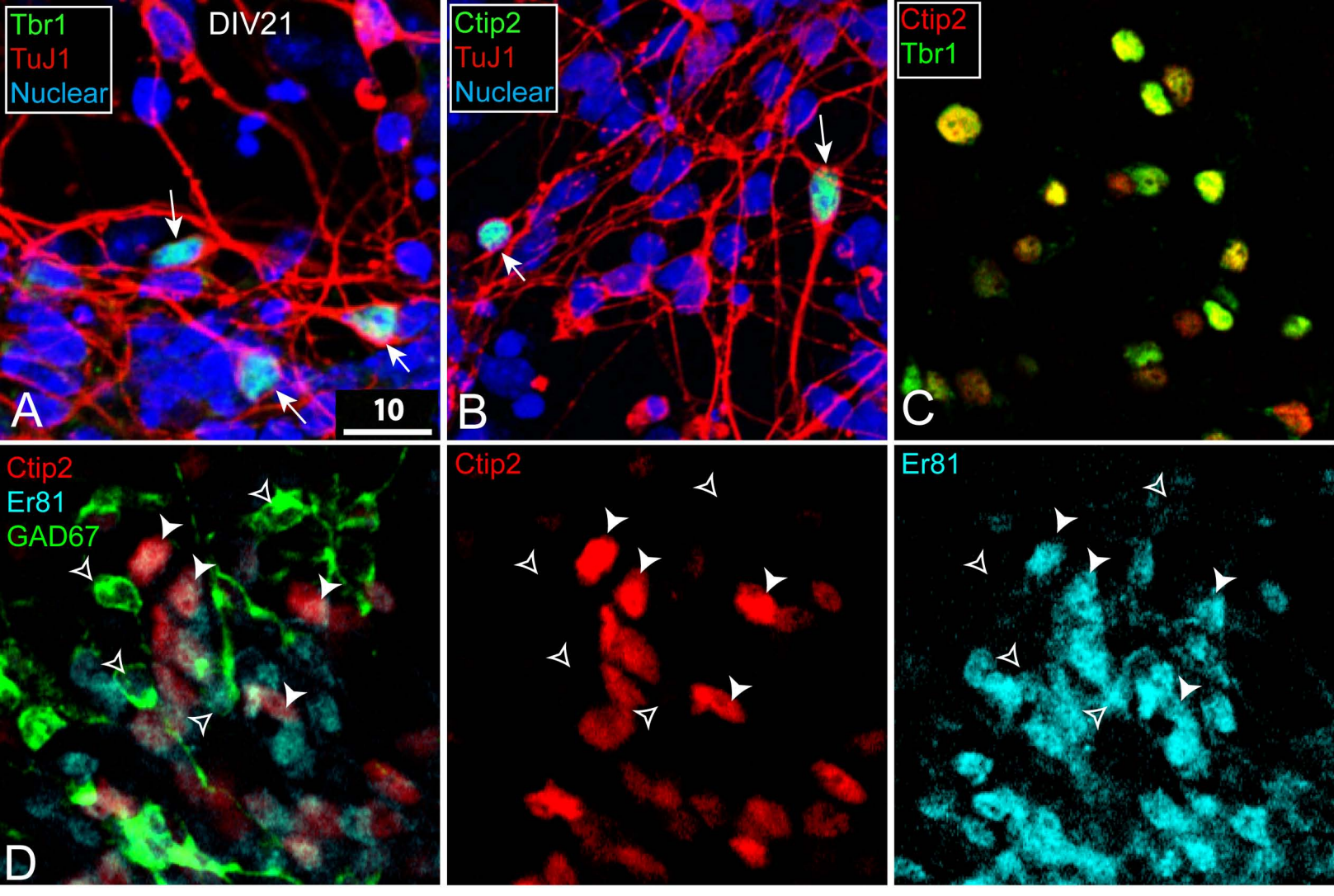
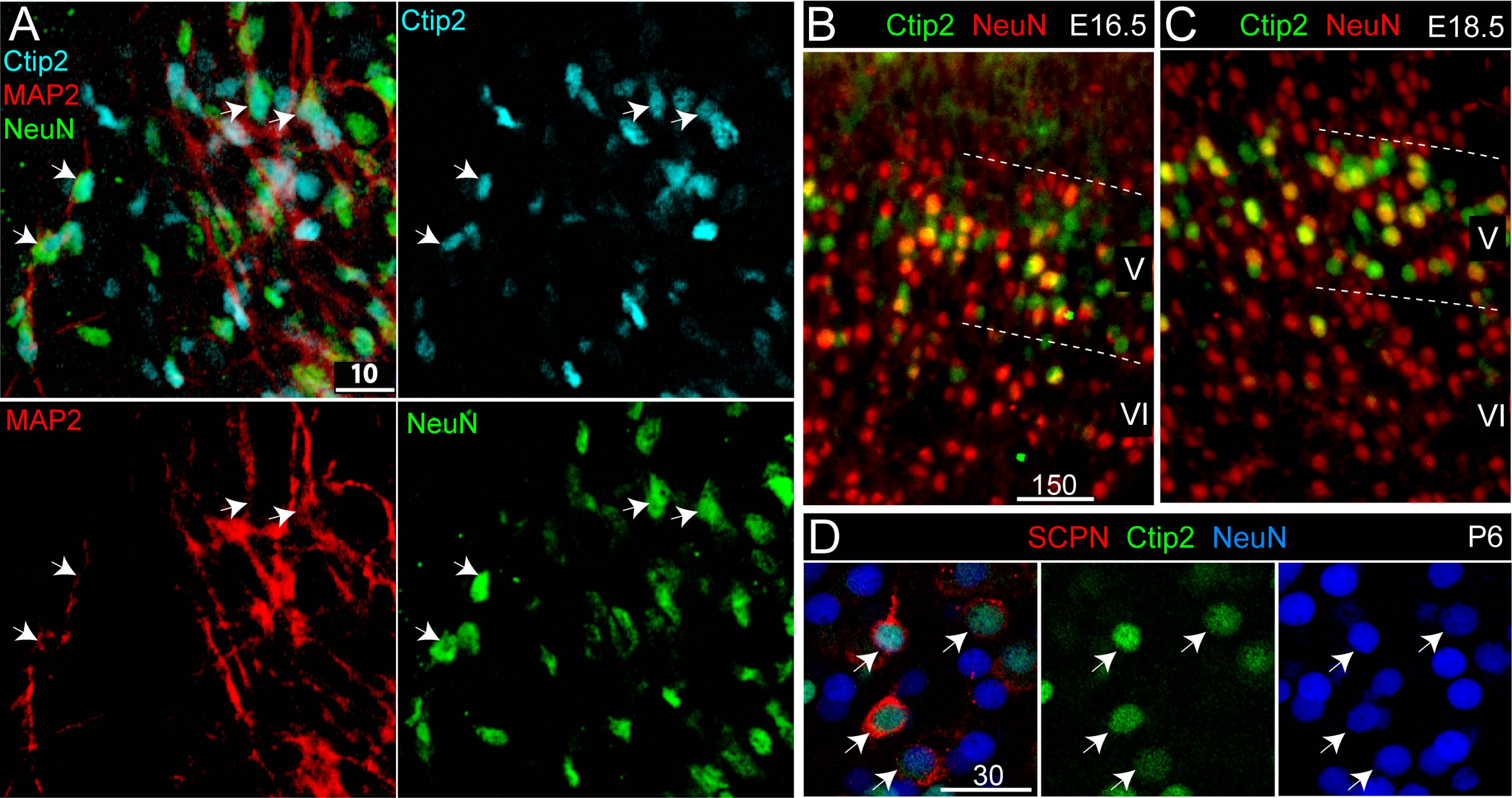
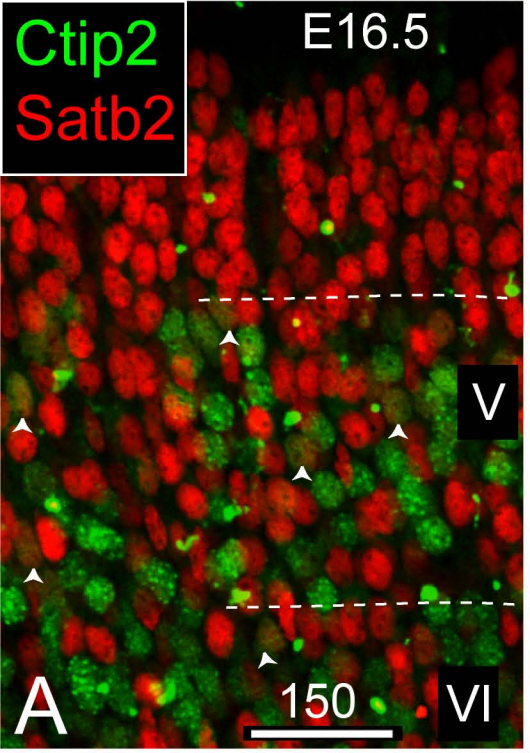
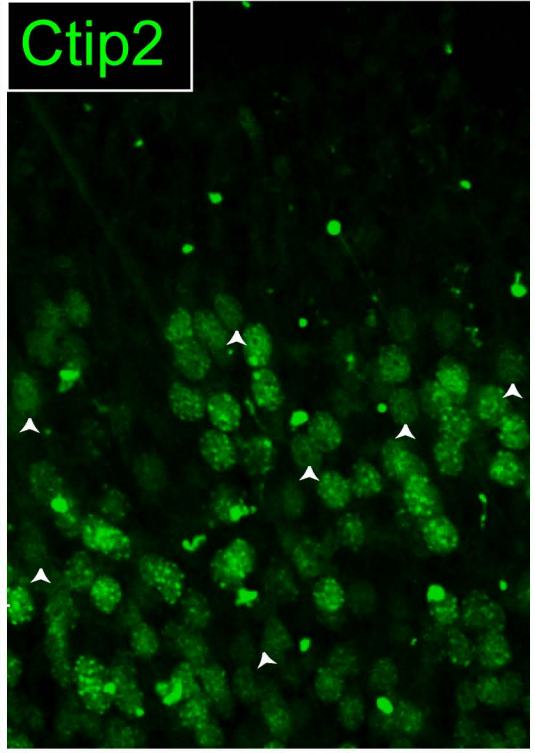
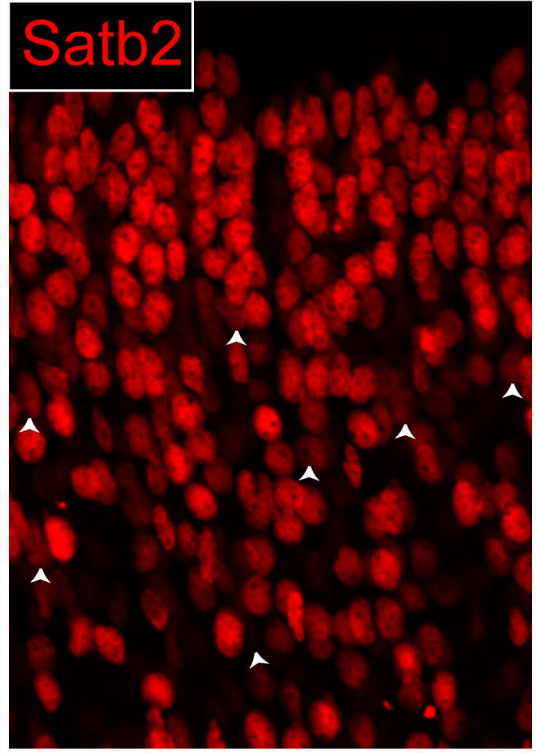
Mash1
L

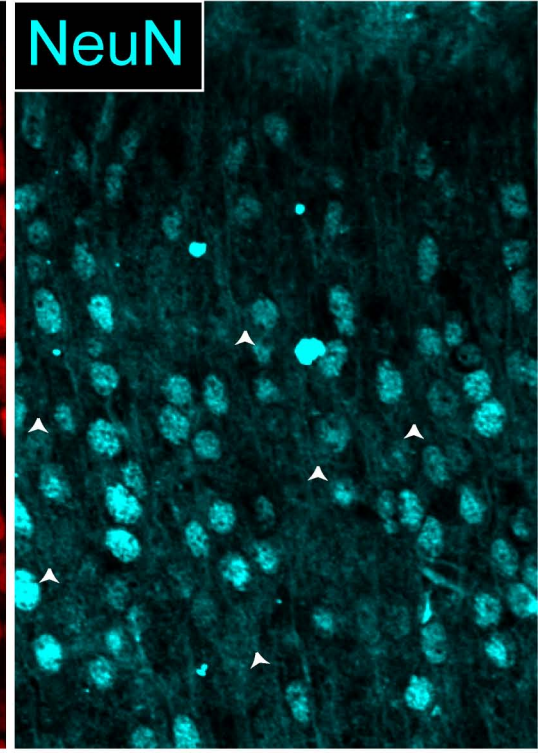

Tbr1
TuJ1
Nuclear
DIV21
A
10
Ctip2
TuJ1
Nuclear
B
Ctip2
Tbr1
C
Ctip2
Er81
GAD67
D
Ctip2
Er81

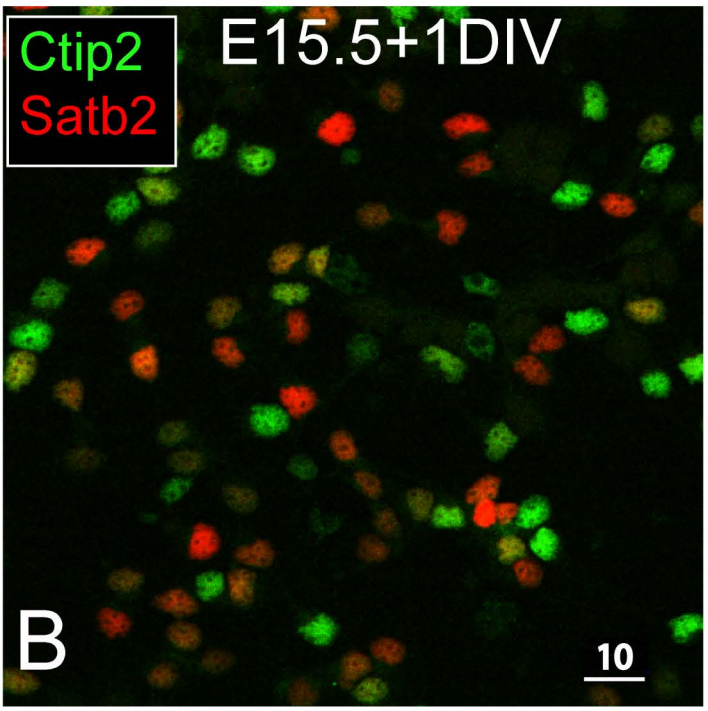

A
Ctip2
MAP2
NeuN
10
Ctip2
MAP2
NeuN
B
Ctip2 NeuN E16.5
V
VI
150
C
Ctip2 NeuN E18.5
V
VI
D
SCPN Ctip2 NeuN P6
30

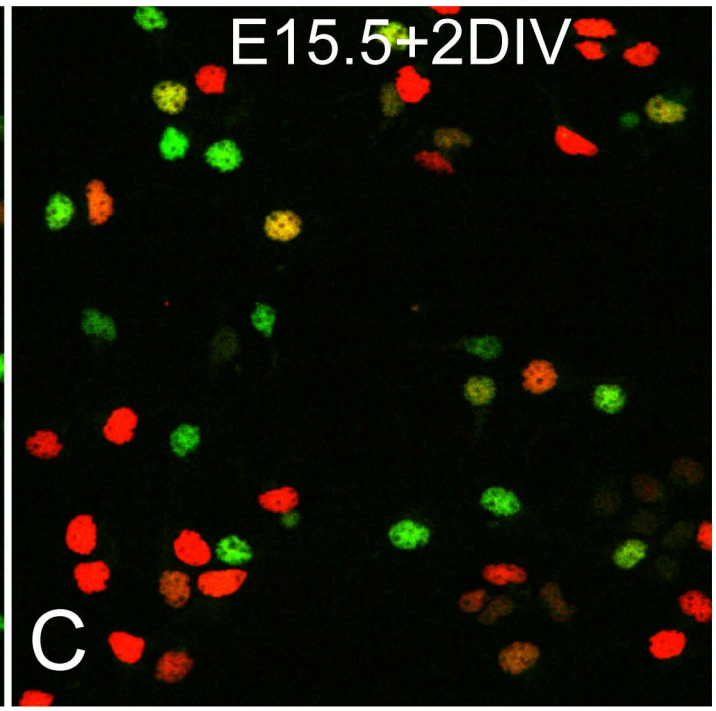

Ctip2
Satb2
E16.5
V
VI
A
150

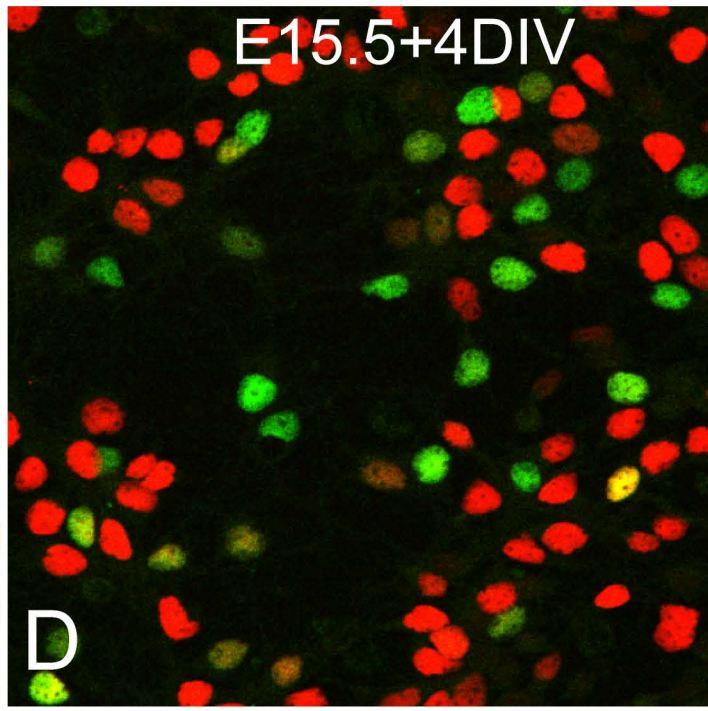

Ctip2

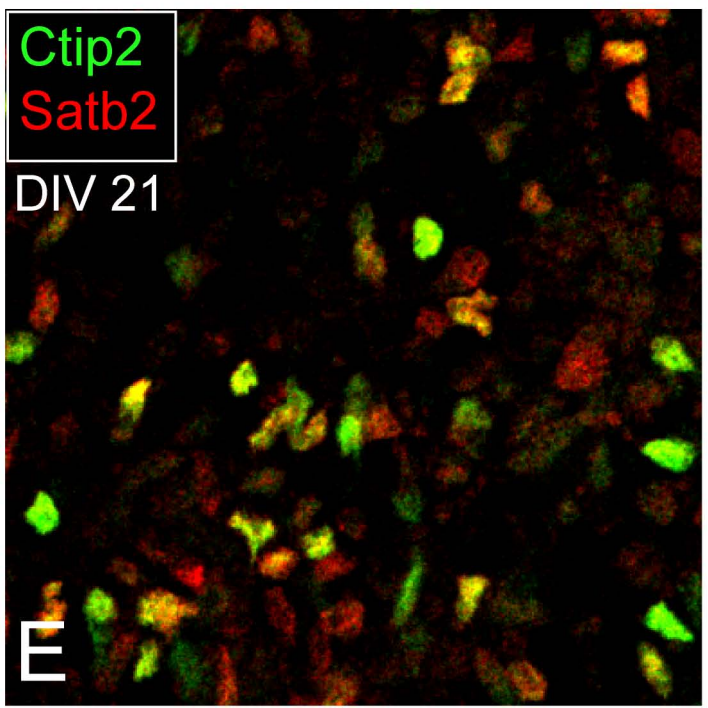

Satb2

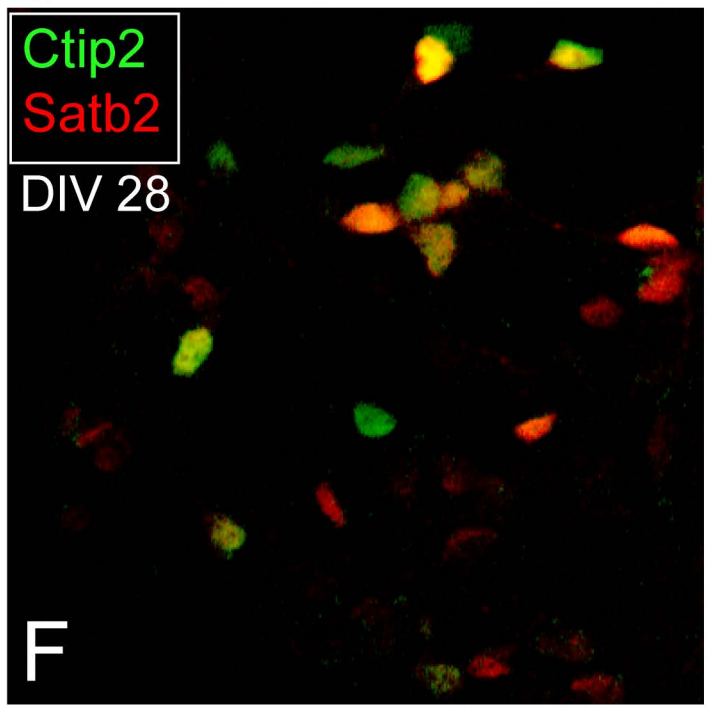

NeuN

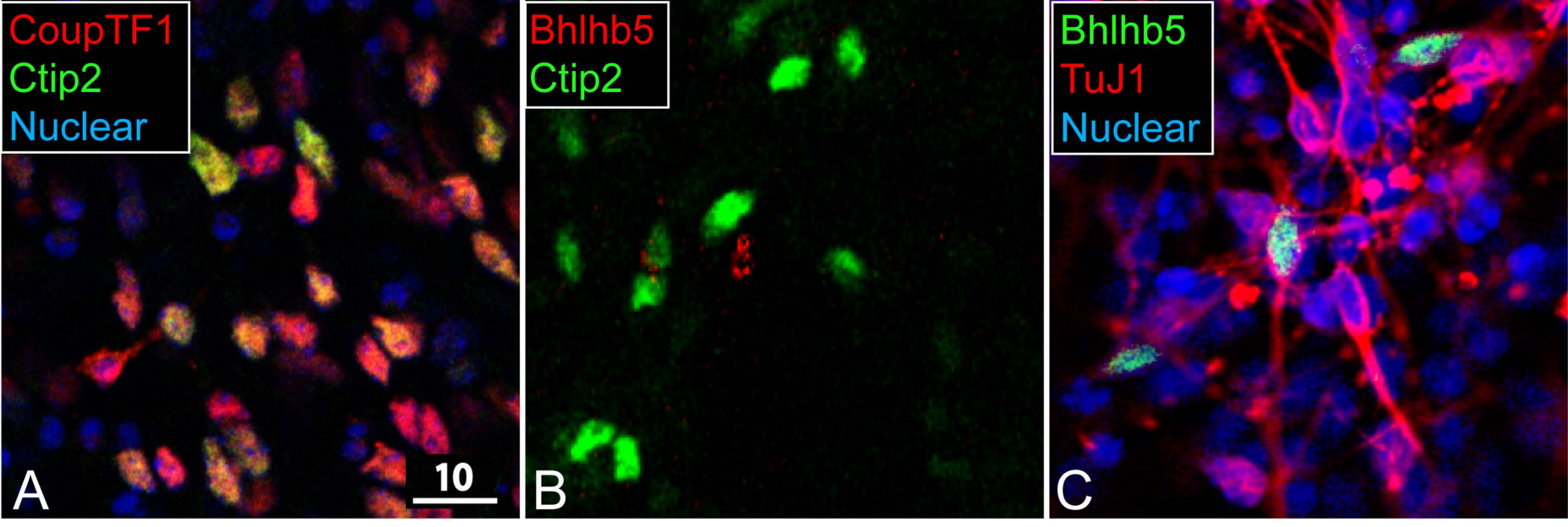

Ctip2
Satb2
E15.5+1DIV
B
10


E15.5+2DIV
C


E15.5+4DIV
D


Ctip2
Satb2
DIV 21
E


Ctip2
Satb2
DIV 28
F


CoupTF1
Ctip2
Nuclear
10
A
Bhlhb5
Ctip2
B
Bhlhb5
TuJ1
Nuclear
C